

Pattern of mismatch repair protein loss and its clinicopathological correlation in colorectal cancer in North India

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Background: To determine the mismatch repair (MMR) protein loss in colorectal cancer (CRC) in north Indian patients and its clinicopathological correlation.

Methods: A prospective study on patients with colorectal cancer from a tertiary level hospital conducted between May 2014 and June 2015. MMR protein loss was determined using immunohistochemistry for MLH1, MSH2, PMS2 and MSH6.

Results: 52 patients (38 male and 14 females) of CRC, with median age of 52.5 years who underwent resection form the study group. 18 (35%) patients were < 50 years of age. Family history of malignancy was present in 3 (6 %) patients. A total of 15 (29%) patients had loss of MMR protein of which 7 (46%) were < 50 years. Most common MMR loss was combined loss of MSH2 + MSH6 [6 (11.5%)] followed by isolated loss of PMS2 [5 (9.6%)]. MMR protein loss was more frequent in patients with right side colon cancer [12 (42%)] compared to left [3 (13%)] (p = 0.033). MMR protein loss was seen in 11 (34%) out of 32 patients fulfilling the revised Bethesda criteria compared to 4 (20%) out of 20 patients who did not fulfil the criteria (p = 0.352).

Conclusion: This study demonstrates high frequency of MMR protein loss in colorectal cancer in north Indian patients which was more common in right colon cancer. Many patients having MMR protein loss do not satisfy the revised Bethesda criteria and would have been missed if selective testing was done. Further research and larger studies are required to validate these findings and develop India specific clinical criteria.

Key words: Colorectal Cancer, Hereditary nonpolyposis colon cancer (HNPCC), Immunohistochemistry, Lynch Syndrome, Microsatellite instability

S Afr J Surg 2018;56(1)

<http://dx.doi.org/10.17159/2078-5151/2018/v56n1a2285>

Introduction

Colorectal cancer (CRC) is the third most common malignancy in the USA with lifetime risk of about 4% and incidence of 40 per 100 000.^{1,2} Initially thought to be an uncommon cancer in India with incidence of 4 per 100 000 but it is increasing over time.^{2,3} The cancer is not uncommon in young age.⁴ It can occur in sporadic form or may be hereditary. Sporadic form is the most common type but genetic and familial associations are known. Risk factors include increasing age, familial predisposition, obesity, smoking and red meat.⁵⁻⁷

Lynch syndrome is the most common genetic type. It is caused by mutations in the mismatch repair (MMR) genes,⁸ which is characterised by autosomal dominant inheritance, predominance for right side cancer and early age of onset.⁹ The diagnosis is clinched by genetic testing which is expensive and not readily available. Hence, before

performing genetic testing, screening tests are performed to select patients most likely to benefit from genetic testing. This can be done by either testing for MMR protein expression using immunohistochemistry (IHC) or by microsatellite instability (MSI) testing using Bethesda markers. Clinical criteria (Amsterdam I and II)^{10,11} were initially suggested to make the clinical diagnosis of Lynch syndrome, but later revised Bethesda guidelines were used to select patients for subjecting to screening tests.¹² But a large number of patients not satisfying the revised Bethesda criteria lack MMR protein expression or have microsatellite instability.¹³⁻¹⁵

There is hardly any data available from India on the given subject. In view of different cancer epidemiology between the west and India, the same cannot be extrapolated here. While cost constraints limit genetic testing to be performed, screening patients using IHC for MMR protein expression

offers an economical alternative to select patients requiring genetic testing.¹⁶ Therefore, the present study has been carried out to find MMR protein loss in colorectal cancer in north India and whether there is any clinicopathological correlation.

Methods

A prospective study on patients with colorectal adenocarcinoma was conducted at Sanjay Gandhi Post Graduate Institute of Medical Sciences from May 2014 to June 2015. The study was approved by the Institute's ethics committee. All patients admitted with the diagnosis of colorectal cancer for surgery in the department of Surgical Gastroenterology formed the study group. Informed consent was taken from all patients. Patients known to have Familial Adenomatous Polyposis (FAP) and cancer arising in the background of inflammatory bowel disease (IBD) were excluded. After informed consent, detailed clinical history, imaging and histological findings were recorded on a defined proforma. IHC was performed as per described standard method.

Immunohistochemistry

Formalin fixed paraffin embedded CRC tissue blocks were sectioned with the help of microtome at 3µm thickness and collected on poly-lysine coated glass slides. Prior to immunostaining, sections were deparaffinised in xylene and rehydrated in an alcohol series (100%, 70% and 30% then with water). Antigen retrieval was done in 1X EDTA (Ethylene diamine tetra acetic acid) buffer (pH 9.0) at 90°C for 30 minutes in microwave. Slides were cooled at room temperature. Endogenous peroxidase activity was blocked by putting the slides in 3% H₂O₂ in methanol for 25 minutes. Slides were incubated with the Anti-MMR antibodies (MLH1- SC56161, Santa Cruz biotechnology, 1:50 dilution, MSH2- SC56163, Santa Cruz biotechnology, 1:150 dilution, PMS2- SC617, Santa Cruz biotechnology, 1:50 dilution, MSH6- EP-49 Dako, Ready-to-use) at room temperature for 2 hours. Slides were washed with Tris-buffer saline (TBS) buffer (3 times). The slides were then incubated with the secondary antibody (Novolink, Maxpolymer detection system 1250, Leica Biosystems, Novacastra, Ready-to-use) for one hour at room temperature followed by washing with TBS three times. Slides were then incubated with 3,3'-Diaminobenzidine (DAB) for 10 minutes (DAB is a chromogenic substrate which stains antigen-antibody sites 'brown'). Counter staining was done by Haematoxylin and eosin. Complete absence of nuclear staining of tumour cells was regarded as loss of MMR protein expression.

Statistical analysis

Continuous data were reported as mean or median and discrete data were reported in percentage. Continuous non-normally distributed variables were compared using 2 tailed student t- test and categorical variables were compared using chi-square test or Fisher's exact test using SPSS version 16.0 (IBM Corporation, Armonk, NY, USA). A *p* value of < 0.05 was considered as significant.

Results

During the study period, a total of 52 patients (38 male and 14 female) of colorectal cancer underwent resection. Median age of the patient was 52.5 (interquartile range 42–60) years. 18 (35%) patients were younger than 50 years. Most common presenting symptom was abdominal pain [*n* = 25 (48%)]. Family history of malignancy was present in 3 (6 %) patients of which two were in first degree relative and one in second degree relative.

Out of 52 patients, 38 (73%) had colon cancer, while 14 (27%) had rectal cancer. Also, 29 (57%) patients had right sided lesion while 23 (43%) had tumour distal to splenic flexure. Two patients had synchronous lesion, while 2 had metachronous lesion. Of these, one had both a synchronous and a metachronous lesion. Histopathological examination showed well differentiated tumour in 16 (31%), moderately differentiated in 10 (19%) and poorly differentiated lesion in 26 (50%) patients. High risk histopathology (poorly differentiated, signet ring type, mucinous type, medullary type, Crohn's like reaction, tumour infiltration of lymphocytes) was present in 27 (52%) patients. Revised Bethesda criteria were fulfilled by 32 (61%) patients. Patient demographics, tumour location and histopathological characteristics are shown in Table 1.

MMR protein loss was found in 15 (29%) patients. Of these, 7 were younger than 50 years. A positive family history was present in only 1 patient. Pattern of MMR loss is depicted in Table 2. Most common MMR protein loss was combined loss of MSH2 + MSH6 seen in 6 (11.5%) followed by isolated loss of PMS2 in 5 (9.6%) patients. Overall, loss of MLH1 was present in 1, loss of PMS2 in 6 (11.5%), loss of MSH2 in 6 (11.5%) and loss of MSH6 in 9 (17.3%) patients.

There was no difference in MMR protein loss based on patient age, gender, tumour differentiation, tumour stage or tumour histopathological characteristics. MMR protein loss was seen more frequently in patients with right side colon cancer [12 (42%)] as compared to left [3 (13%)] which was statistically significant (*p* = 0.033). There was no statistical difference in MMR protein loss even between early stage (Stage I/II) compared to late stage (III/IV) tumours. The difference was also not significant between low risk tumours (well/moderately differentiated and no high risk HPE features) compared to high risk tumours (poorly differentiated and high risk HPE features) (Table 3).

Out of 32 patients fulfilling the revised Bethesda criteria, MMR protein loss was seen in 11 (34.4%) patients which was similar to patients not fulfilling the revised Bethesda criteria (*p* = 0.266).

Discussion

The present study represents one of the first few studies from north India to determine the loss of MMR protein expression in colorectal cancer. IHC was used because of its high sensitivity and specificity in detecting microsatellite instability. The sensitivity of IHC to detect MMR loss is 65–70% with the use of 2 antibodies (MLH1, MSH2) which

Table 1. Patient Demographics, tumour location, histopathological characteristics and MMR expression in patients of colorectal cancer.

	MMR expressed (n = 37)	MMR Loss (n= 15)	P value
Gender			0.731 [^]
Male	26 (70.3 %)	12 (80%)	
Female	11 (29.7%)	3 (20%)	
Age (Years) (IQR)	53 (45-60)	50 (40-60)	0.469
Age			0.245**
< 50 Years	11 (29.7 %)	7 (46.7 %)	
≥ 50 Years	26 (70.3%)	8 (53.3%)	
Tumour Proximal to Splenic Flexure (n = 29)	17 (45.9 %)	12 (80%)	0.033* [^]
Synchronous Lesion	1 (2.7 %)	1 (6.7%)	0.498 [^]
Metachronous Lesion	0 (0 %)	2 (13.3%)	0.079 [^]
Family H/O Malignancy	2 (5.4%)	1 (6.7%)	1.0 [^]
Differentiation			0.221**
Well differentiated	13 (35.1%)	3 (20%)	
Moderately differentiated	5 (13.5%)	5 (33.3%)	
Poorly differentiated	19 (51.4%)	7 (46.7%)	
High risk HPE	20 (38.4%)	7 (46.7%)	0.629**
Mucinous Type	6 (16.2%)	3 (20%)	1.0 [^]
Signet Ring Cell	6 (16.2%)	1 (6.7)	0.658 [^]
Tumour infiltration of lymphocytes	6 (16.2%)	1 (6.7%)	0.658 [^]
Revised Bethesda Criteria			0.352 [^]
Fulfilled	21 (56.8%)	11 (73.3%)	
Not-fulfilled	16 (43.2%)	4 (26.7%)	

*Statistically Significant

[^] Fisher's Exact test

** Chi- Square test

Note: Medullary pattern and Crohn's like reaction were not present in any of the tumours

is further increased to 90–92% with use of all four antibodies (MLH1, MSH2, MSH6, PMS2).

The male to female distribution was 3:1 in this study compared to 1.2–1.5:1 in the west.¹⁷ It was 2.1:1 in a study reported from south India.¹⁸ In another study from south India comprising of right colon cancer only, male to female ratio was 2.4:1.¹⁹ In yet another study from Rajasthan (west India), the male to female ratio was 1.7:1.²⁰ This shows an overall higher male to female ratio in India with large regional variations within India.

Thirty-five percent of our patients were under 50 years compared to only 10% in USA.¹⁷ A large regional variation in the age distribution is seen in India with cases less than 50 years representing 20–50% of the total cases.¹⁸⁻²¹ Colorectal cancer in India thus appears to be more frequent in younger patients compared to the west, with large regional variations. This may be because of different environmental factors or may represent a different genetic predisposition.

MMR protein loss of 29% in the present study is higher than previously reported. A study from the UK reported MMR protein loss of 21% while another from Memorial

Sloan-Kettering Cancer Centre reported it as 19%; other studies from the west have reported MMR protein loss from 15–21%.²²⁻²⁴ Wenxue Zhi et al. from China reported MMR loss in 7% patients.²⁵ A recent study from north India by Dubey et al. reported MSI- high in 22% patients.²⁶ Earlier, two studies from India by Pandey et al.²⁷ and Malhotra et al.²⁸ have reported MMR protein loss from 17.8–19.9%. But both had reported their study with only 2 antibodies, i.e. MLH1 and MSH2 in contrast to the present study where all four antibodies were tested (MLH1, MSH2, MSH6, PMS2). MLH1 loss was reported in 15.7% patients by Pandey et al. and in 13.3% by Malhotra et al. compared to only 1.9% in this series. Such low rates of MLH1 loss could not be explained.

Lynch syndrome accounts for about 3-5% of colorectal cancer which is caused by germline mutation in the MMR gene. Isolated loss of MLH1 is usually the result of promoter hypermethylation preventing its expression and hence is usually sporadic. The present study has no isolated MLH1 loss which may suggest a high proportion of Lynch syndrome amongst patients with MMR protein loss.

MMR protein loss is more commonly seen in right sided

Table 2. Pattern of MMR loss in patients with colorectal cancer.

MMR Protein Lost	No. of Patients (n = 52)
MSH2 + MSH6	6 (11.5%)
PMS 2 Only	5 (9.6%)
MSH 6 only	3 (5.8%)
MLH1 + PMS2	1 (1.9%)

Table 3. MMR protein loss based on tumour stage and tumour characteristics.

	Total (n= 52)	MMR expressed (n = 37)	MMR Loss (n= 15)	P value
Stage				0.213**
I	2 (3.8%)	2 (5.4%)	0	
II	25 (48.1%)	20 (54.1%)	5 (33.3%)	
III	20 (38.5%)	13 (35.1%)	7 (46.7%)	
IV	5 (9.6%)	2 (5.4%)	3 (20%)	
Stage				0.08^
Early (I/II)	27 (51.9%)	22 (59.5%)	5 (33.3%)	
Late (III/IV)	25 (48.1%)	15 (40.5%)	10 (66.7%)	
Tumour Characteristic (n =29)				0.474^
Low Risk Tumours	14 (48.3%)	11 (52.4%)	3 (37.5%)	
High Risk Tumours	15 (51.7%)	10 (47.6%)	5 (62.5%)	

^ Fisher's Exact test
** Chi- Square test

tumours compared to left sided and rectal tumours as was evident in this series as well. This is similar to studies from other parts of the world,^{24-26,29} however, Ravikanth et al.³⁰ reported MSI-H status to be present most commonly in rectal cancer (66%).

MMR protein testing using IHC is a less costly, rapid method compared to MSI testing, having similar sensitivity with the use of all 4 antibodies. It also directs further germline mutational analysis.²² Patients having MMR protein loss may be suspected to have Lynch syndrome, and warrant genetic testing. A major hurdle in this approach is lack of facilities for genetic counselling and testing. Even at places where such facility is available, IHC testing for MMR protein is not readily available. One of the factors for non-availability of a relatively economical IHC testing is lack of data regarding the prevalence of MMR protein loss in India; hence, its importance is not understood. A 29% loss of MMR protein needs no further emphasis on the importance of MMR protein testing.

Out of 15 patients having MMR protein loss, 4 (27%) patients did not meet the revised Bethesda criteria. Hence, one-fourth MMR protein loss would have been missed if testing was done based on the above criteria alone. At the same time, 4 (20%) patients out of 20 who did not satisfy the revised Bethesda criteria had MMR protein loss. Hence, if testing had been done solely based on revised Bethesda criteria, one-fifth of patients carrying the MMR protein loss would have been

missed. Therefore, short of universal IHC testing, clinical criteria specific for Indian patients are required before genetic testing for patients of colorectal cancer.

The strength of the study lies in its prospective nature. However, the study lacks genetic analysis which is done to actually determine the patients suffering from Lynch syndrome. A correlation with MSI testing is also missing from the study.

Conclusion

This study demonstrates high frequency of MMR protein loss in colorectal cancer in north Indian patients which was more common in right colon cancer. Many patients with MMR protein loss do not satisfy the revised Bethesda criteria and would be missed if selective testing were done. Further research and larger studies are required to validate these findings and develop India specific clinical criteria.

Conflict of Interest: Authors declare that they have no conflict of interest to declare.

Funding: The study was funded by intra-mural grant by SGPGIMS, Lucknow.

Acknowledgement: The authors gratefully acknowledge Professors VK Kapoor, Anu Behari, Rajneesh Kumar Singh

and Dr Anand Prakash for contributing case material for this study.

Note: Dr Ashok Kumar and Dr Mayank Jain have contributed equally in the study.

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